

# Chapter 5

## **A comparative study of inosine triphosphatase activity in fresh erythrocytes and dried blood spots**

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## Abstract

Inosine triphosphatase (ITPase) is one of the enzymes involved in thiopurine metabolism. Polymorphisms in the *ITPA* gene, resulting in a decreased activity of ITPase, are associated with adverse drug reactions during thiopurine therapy. Measuring ITPase activity is therefore of utmost importance. We compared measurement of ITPase activity in fresh erythrocyte lysates and eluates of dried blood spots (DBS). Upon storage up to 2 years at room temperature a fast decrease of ITPase activity in DBS was observed. The residual activity declined from 70% of the activity measured in fresh erythrocyte lysate 3 days after spotting to ~5% of after 2 years. Patients with an activity lowering polymorphism in the *ITPA* gene could still be detected in DBS after longer periods of storage at room temperature, using DBS of patients with a normal wild type activity, which were stored over the same period as reference. However, routine determination of ITPase activity in fresh erythrocyte lysates is preferred.

## Introduction

Thiopurines are used as cytotoxic drugs in the treatment of a variety of autoimmune mediated and lymphoproliferative diseases, e.g. inflammatory bowel disease (IBD), rheumatic diseases, dermatological conditions, sarcoidosis and childhood acute lymphatic leukemia (ALL)<sup>1</sup>. The metabolism of thiopurines is a complex network of activation and deactivation, a tight balance being essential for therapeutic efficacy and avoidance of adverse drug reactions (ADR)<sup>2-4</sup>.

One of the enzymes involved in thiopurine metabolism is inosine triphosphatase (ITPase). ITPase catalyses the conversion of inosine-5'-triphosphate (ITP) and deoxyinosine-5'-triphosphate (dITP) into the respective monophosphate esters. A concise outline of this pathway is shown in Figure 5.1A. The *ITPA* gene is a polymorphic gene<sup>5</sup>. The effects of these polymorphisms on ITPase activity varies, as some have no influence, others result in a very low enzyme activity in the homozygous state. Deficiency of ITPase results in accumulation of intracellular ITP. Under normal physiological conditions ITPase deficiency does not lead to a clinical phenotype, so far it is considered a benign condition<sup>6,7</sup>.

In the last decade more insight has become available on the significance of ITPase in thiopurine metabolism. There is an increasing number of reports on the occurrence of ADR in combination with polymorphisms in the *ITPA* gene<sup>8,9</sup>. Recently we demonstrated that ITPase can hydrolyze 6-thioITP (TITP), the triphosphate which is formed through the phosphorylation of 6-thioinosine monophosphate (TIMP) by the intracellular mono- and diphosphate kinases<sup>10</sup>. This implies that ITPase is involved in thiopurine homeostasis (Figure 5.1B). In addition, it is generally assumed that thiopurine-S-methyltransferase (TPMT) is capable of methylating 6-thioITP, resulting in the formation of methyl-6-thioITP (MTITP). The clinical symptoms associated with accumulation of TITP and MTITP to cytotoxic levels are flu-like symptoms, rash and pancreatitis<sup>9,11</sup>.

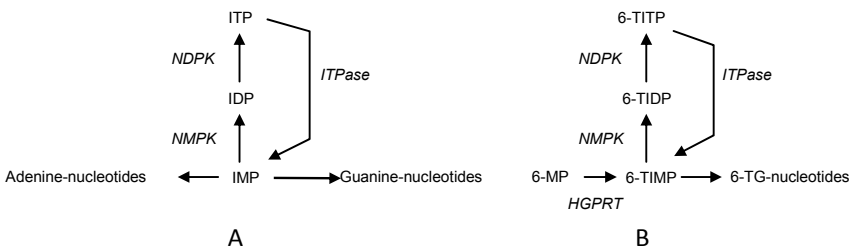


Figure 5.1 Role of ITPase in purine metabolism (simplified scheme), panel A physiological metabolism, panel B thiopurine metabolism. Abbreviations used: HGPRT: hypoxanthine-guanine phosphoribosyl transferase, IM/D/TP: inosinemono/di/triphosphate, ITPase: inosine triphosphatase, 6-MP: 6-mercaptopurine, NM/DPK: nucleotide mono/diphosphate kinase, 6-TG: 6-thioguanosine, 6-TIM/D/TP: 6-thioinosinemono/di/triphosphate.

In erythrocytes a relatively high activity of ITPase is present and therefore it is easily measured in erythrocyte lysates<sup>5</sup>. However, shipment of blood samples, both national and international, is subject to tight regulations and sometimes very cumbersome. For ease of transportation the use of dried blood spots (DBS) can be advocated. This urged us to study the feasibility of dried blood spots as a possible material for ITPase measurement.

In neonatal screening programs DBS are in use since the early 70's, phenylalanine being the first analyte measured in DBS<sup>12</sup>. In the following years the application of DBS was extended by measuring other metabolites and more complex molecules like steroids and thyroid hormones. In addition DBS proved to be suitable for enzyme diagnostics, particularly for lysosomal storage disorders<sup>13</sup>. Jacomelli et al. reported the use of DBS to measure enzymes involved in purine metabolism<sup>14</sup>. Recently Tomkova et al. described a capillary electrophoresis method for the determination of ITPase activity in DBS, proposing the use of DBS for routine measurement of ITPase activity<sup>15</sup>. We compared the ITPase activity in fresh erythrocyte lysates with the ITPase activity in DBS stored over longer periods, using samples from patients having a normal genotype and patients carrying activity lowering polymorphisms in the *ITPA* gene.

## Materials and methods

IMP, ITP, hypoxanthine, hemoglobin and DTT were purchased from Sigma (Zwijndrecht, The Netherlands). Perchloric acid was purchased from J.T. Baker (Deventer, The Netherlands).  $MgCl_2$ ,  $K_2CO_3$  and  $(NH_4)H_2PO_4$  and  $(NH_4)_2HPO_4$  were purchased from Merck (Amsterdam, The Netherlands). Tetrabutylammonium bi-sulphate (TBS) and Trizma<sup>®</sup> base (Tris) were purchased from Fluka (Zwijndrecht, The Netherlands). Methanol (HPLC-grade) was purchased from Biosolve (Valkenswaard, The Netherlands). Ultrapure water was prepared in house (Advantage system, Millipore, Etten-Leur, The Netherlands) and used for the preparation of all solutions.

### Samples

DBS were made from EDTA anticoagulated blood samples obtained for routine diagnostic purposes shortly after receipt of the sample. Blood was spotted onto newborn screening (NBS) cards and stored at room temperature. Erythrocyte lysates were made as previously described<sup>16,17</sup>. Lysates were stored at  $-80^\circ C$  prior to analysis.

### ITPase assay

The ITPase assay was performed as previously described with minor modifications for the measurement in DBS<sup>16,17</sup>. DBS were incubated with 700  $\mu l$  100mM Tris solution

pH 8.5 during 30 min at room temperature. The DBS eluate was transferred to a reaction vial and centrifuged for 10 min at 11.000  $\times g$ . 165  $\mu$ l of the eluate was used to measure ITPase activity. All measurements were performed in duplicate. Reaction conditions were as described previously for fresh erythrocyte lysates<sup>16,17</sup>. Chromatographic separations were performed using an ion-pair reversed phase protocol (phosphate buffer 0.5 mM pH 7.0 with TBS) on an Alliance binary HPLC system (Waters, Etten-Leur, The Netherlands) coupled with a Jasco 2077 multi-wavelength UV/Vis detector (De Meern, The Netherlands)<sup>16</sup>. Data were sampled and analyzed using Totalchrom data acquisition and handling software (Perkin Elmer, Groningen, The Netherlands).

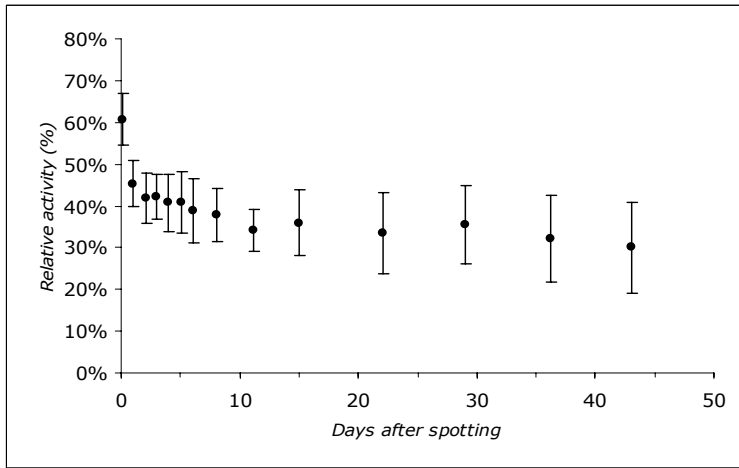
### Determination of DBS haemoglobin concentration

Hemoglobin (Hb) in fresh erythrocyte lysates was measured using a Coulter LH 750 hematology analyzer (Beckman Coulter, Woerden, The Netherlands). Hemoglobin in DBS lysates was measured using a Cary 50 Bio UV-Vis spectrophotometer, equipped with a Cary 50 MPR plate reader unit (Varian Scientific Instruments, Middelburg, The Netherlands). 110  $\mu$ l DBS eluate was diluted with 220  $\mu$ l 100mM Tris buffer pH 8.5 and the Hb concentration in the DBS lysate was measured in triplicate and the absorbance at 416 and 640 nm was recorded. Corrected readings were used to calculate the Hb concentration against a calibration curve of hemoglobin in 100 mM Tris pH 8.5<sup>18</sup>.

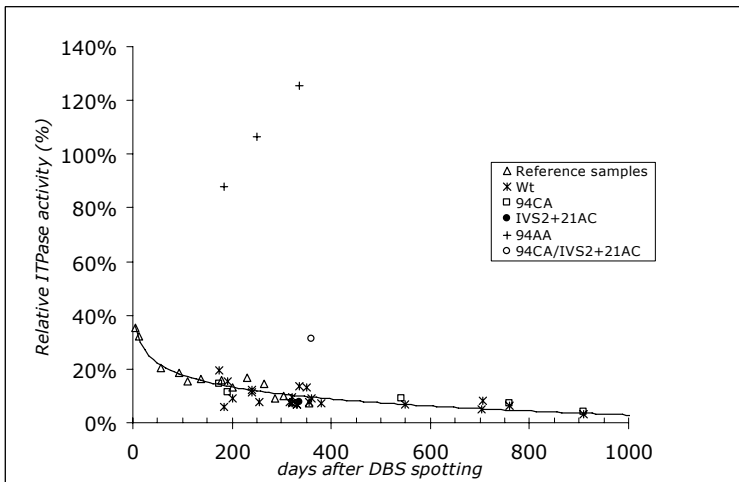
## Results

The stability of ITPase activity in DBS was studied in the following experiment. Freshly drawn EDTA anticoagulated blood samples were divided in two portions. The first portion was used to isolate erythrocytes. The second portion was spotted onto NBS cards. The ITPase activity measured in erythrocyte lysates was set at 100%. For a period of 45 days the ITPase activity in DBS was measured at regular intervals. Immediately after spotting and drying the ITPase activity in DBS was 70% of the activity measured in erythrocyte lysates. The ITPase activity declined to 10-20% within 2 months after spotting (Figure 5.2).

*ITPA* is a polymorphic gene, and hence there is a great diversity in ITPase activity in the general population<sup>5,17</sup>. Carriers of polymorphisms are easily identified by measuring ITPase activity in fresh erythrocyte lysates<sup>16</sup>. To test the possibility to discriminate between Wt ITPase activity and carriers of known polymorphisms using DBS, we measured the ITPase activity in DBS in patients with known genotypes. DBS between 165 and 910 days old were tested, using DBS of Wt patients spotted at the same time as reference (Figure 5.3). Table 5.1 shows that the relative activity in both the polymorphic DBS and the wild type DBS is comparable, and that carriers of enzyme activity lowering polymorphisms could be detected.



**Figure 5.2** ITPase activity in DBS of healthy controls during time, expressed as percentage of the activity in fresh erythrocyte lysate.



**Figure 5.3** Evaluation of influence of storage time at room temperature on ITPase activity in DBS of randomly selected patients with normal ITPase activity and genotyped patients. Relative ITPase activity is calculated as a percentage of the ITPase activity in fresh erythrocyte lysates.

**Table 5.1** ITPase activity in DBS correlated to DNA polymorphism and time after spotting.

DNA polymorphism	Lysate activity (mmol/mmol Hb/hr)	Days after spotting	DBS activity (mmol/mmol Hb/hr)	Rel. activity (%)
Wt	4.46	165	0.87	19.5
c.94C→A	2.36	165	0.34	14.5
Wt	4.18	180	0.65	15.5
c.94C→A	1.44	180	0.16	11.2
Wt	3.71	185	0.22	5.8
c.94C→A / c.94C→A	0.03	185	0.03	88.1
Wt	4.96	250	0.38	7.6
c.94C→A / c.94C→A	0.04	250	0.04	106.5
Wt	7.80	335	1.05	13.1
IVS2+21A→C	2.00	335	0.15	7.6
c.94C→A / c.94C→A	0.03	335	0.04	125.4
Wt	4.38	360	0.9	9.0
c.94C→A / IVS2+21A→C	0.26	360	0.08	31.1
Wt	5.21	550	0.36	6.8
c.94C→A	2.44	550	0.22	9.0
Wt	4.79	760	0.31	6.5
c.94C→A	1.90	760	0.14	7.2
Wt	4.61	910	0.14	3.1
c.94C→A	1.19	910	0.05	4.1
Reference range	3.0 – 11.0			

## Discussion

From the results of our experiments it is obvious that measurement of ITPase activity in DBS is not as straightforward as supposed by Tomkova et al.<sup>15</sup>. The activity of ITPase in freshly prepared DBS is less than 70% of the lysate activity. Furthermore the decrease of activity in DBS is fast. Within 3 days after spotting the relative activity has declined to 30%. This hampers the use of DBS for the determination of ITPase activity as on the average ordinary mail delivery will take 2 – 3 days. In the case of patients with an activity of ITPase in fresh erythrocyte lysate at the lower end of the reference range, which in our laboratory is 3.0 - 11.0 mmol IMP/mmol Hb/hr (based on Wt genotype), the measured activity in the DBS will easily decrease to intermediate values. Given the fact that intermediate values of ITPase are associated with ADR, it is obvious that using DBS may result in erroneous interpretation<sup>9,19,20</sup>. This may lead to suboptimal dosing of thiopurine medication.

Although the difference between normal and low activities still can be made, the older the DBS gets, the more difficult it will be. As can be seen from Table 5.1 patients homozygous for the c.94C→A polymorphism are still easily detected, because of the very low ITPase activity, both in fresh erythrocyte lysate as well as in DBS. However, more specifically the difference between intermediate values, due to heterozygosity for *ITPA* polymorphisms, and normal activities will not be apparent. An accompanying control DBS might overcome this problem, although one must be aware that ~10% of

the Caucasian population is heterozygous for an ITPase activity lowering polymorphism<sup>5</sup>.

## Conclusion

The results of our experiments demonstrate that ITPase activity can be measured in DBS without technical difficulties. We have demonstrated that immediately after spotting there was already a 30% loss of ITPase activity in DBS, which declined further and very rapidly to 30% residual activity in three days. The idea of DBS is to provide convenient mail order diagnostics. Considering the fact that regular (inter)national mail delivery requires 2-3 days at the minimum, reliable diagnostic measurement in DBS does not seem realistic. Therefore we strongly advocate measurement of ITPase activity in fresh erythrocyte lysates.



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